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A novel eight amino acid insertion contributes to the hemagglutinin cleavability and the virulence of a highly pathogenic avian influenza A (H7N3) virus in mice

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ABSTRACT

In 2012, an avian influenza A H7N3 (A/Mexico/InDRE7218/2012; Mx/7218) virus was responsible for two confirmed cases of human infection and led to the death or culling of more than 22 million chickens in Jalisco, Mexico. Interestingly, this virus acquired an 8-amino acid (aa)-insertion (..PENPK-**DRKSRHRR**-TR/GLF) near the hemagglutinin (HA) cleavage site by nonhomologous recombination with host rRNA. It remains unclear which specific residues at the cleavage site contribute to the virulence of H7N3 viruses in mammals. Using loss-of-function approaches, we generated a series of cleavage site mutant viruses by reverse genetics and characterized the viruses in vitro and in vivo. We found that the 8-aa insertion and the arginine at position P4 of the Mx/7218 HA cleavage site are essential for intracellular HA cleavage in 293T cells, but have no effect on the pH of membrane fusion. However, we identified a role for the histidine residue at P5 position in viral fusion pH. In mice, the 8-aa insertion is required for Mx/7218 virus virulence; however, the basic residues upstream of the P4 position are dispensable for virulence. Overall, our study provides the first line of evidence that the insertion in the Mx/7218 virus HA cleavage site confers its intracellular cleavability, and consequently contributes to enhanced virulence in mice.

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Introduction

Influenza A viruses including 16 of the 18 hemagglutinin (HA) subtypes and 9 of the 11 neuraminidase (NA) subtypes primarily have their natural reservoir in wild aquatic birds (Webster et al., 2006). Although infection in their natural hosts is typically asymptomatic, influenza viruses, especially the H5 and H7 subtypes, can spread to domestic poultry and cause mild to severe disease, resulting in significant economic losses (Kalthoff et al., 2010). In addition, H5 and H7 subtype viruses have repeatedly jumped the species barrier from poultry to humans. The first well-documented case of human infection by an avian influenza virus through direct contact with infected birds occurred in 1996 (Kurtz et al., 1996). Since then, multiple subtypes of avian influenza viruses including H5, H6, H7, H9 and H10 have caused infection in humans, with severe and fatal disease possible with selected strains (Ozawa and Kawaoka, 2013; Subbarao and Joseph, 2007; Yuan et al., 2013). The increasing number of human influenza cases caused by avian influenza viruses underscores the need to better evaluate the risk of virulence, transmissibility and adaptation of these subtype viruses in mammalian hosts.

During June–August 2012, outbreaks of a highly pathogenic avian influenza A (HPAI) H7N3 subtype virus were reported in poultry farms throughout Jalisco State, Mexico, resulting in the deaths of approximately 22 million birds through either disease or culling (FAO, 2012). During the H7N3 outbreaks in Mexico, two poultry farm workers with direct contact to infected poultry developed conjunctivitis without fever or respiratory symptoms. Influenza H7N3 virus, A/Mexico/InDRE7218/2012 (Mx/7218) was isolated from one of the human cases (Lopez-Martinez et al., 2013; CDC, 2012) and found to be closely related to the circulating avian HPAI virus A/Chicken/Jalisco/CPA/2012 (H7N3) (Lopez-Martinez et al., 2013). Compared to other H7 viruses isolated from the outbreaks in poultry farms in the Americas in recent years, the 2012 H7N3 viruses isolated in Mexico are of special interest because the virus has demonstrated an ability to transmit to humans through direct contact or close proximity with infected animals (Lopez-Martinez et al., 2013). Experimentally, the closely related avian A/Chicken/Jalisco/CPA/2012 (H7N3) virus caused severe clinical disease and high mortality in chickens, similar to results reported for

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other HPAI viruses (Kapczynski et al., 2013). Pathogenesis studies in mammalian models revealed that the Mx/7218 virus replicated efficiently in the ferret respiratory tract and caused lethal infection in mice (Belser et al., 2013), which is in contrast to pathogenesis results observed for the lesser virulent 2004 H7N3 virus isolated from a human conjunctivitis case in British Columbia, Canada (Belser et al., 2007).

The influenza HA displayed on the viral envelope is responsible for both receptor binding and fusion between the host endosome and the viral membrane (Wiley and Skehel, 1987). The HA is synthesized as the polypeptide precursor HA0, which requires post-translational cleavage at the conserved arginine (R329, H3 numbering) located at the HA1/HA2 boundary. Activation of the fusion protein results in release of the fusion peptide and subsequent insertion into the target host cell membrane (Wiley and Skehel, 1987). This is a process that is dependent on the HA cleavage efficiency of host proteases. Structurally, the HA is organized in the viral membrane as a homotrimer composed of a globular head domain, a stem domain, and the HA cleavage site – the latter of which is projected away from the stem domain and exposed into solution, where it is accessible to proteases (Steinhauer, 1999). Depending on the primary sequences around the HA1/HA2 boundary, the proteases responsible for cleavage and the locations at which the cleavage occurs may vary (Steinhauer, 1999). Most human and low pathogenic avian influenza viruses, which possess a single arginine (R) residue at the HA1/HA2 boundary, are cleaved by extracellular or membrane-bound airway trypsin-like proteases. Such proteases include tryptase Clara found in the respiratory tract (Chen et al., 2000; Kido et al., 1992) or the recently identified serine proteases, TMPRSS2 and HAT, from the human airway epithelium (Bottcher et al., 2006). In contrast, HPAI H5 or H7 subtype viruses typically possess a stretch of R or lysine (K) residues at the HA cleavage site and can be recognized by ubiquitous subtilisin-like intracellular proteases. Such proteases include furin and PC6, both of which are specific for a minimal consensus sequence R-X-K/R-R for cleavage and can lead to systemic virus spread and increased virulence (Hosaka et al., 1991; Horimoto and Kawaoka, 1995; Horimoto et al., 1995). Furthermore, the cleavability of the HA protein can also be affected by how accessible the cleavage site is to the proteases. For example, the removal of a glycan located in close proximity to the HA cleavage site is necessary for HA intracellular cleavage of an avian H5N1 virus containing the R-X-K/R-R motif (Kawaoka et al., 1984; Kawaoka and Webster, 1989). In addition to removing steric hindrance posed by glycans, the structural accessibility of the HA cleavage site can also be improved by projecting it further away from the trimer stem surface as seen in some H7 viruses, in which an insertion acquired by nonhomologous recombination can facilitate cleavage by further exposing the cleavage site to the solvent (Hirst et al., 2004; Orlich et al., 1994).

Unlike most HPAI viruses, the Mexico H7N3 virus acquired a unique 8-amino acid (aa) insertion through nonhomologous recombination with host 28S rRNA (Kapczynski et al., 2013; Maurer-Stroh et al., 2013). In general, HA insertions acquired by nonhomologous recombination are rare, but have been previously observed among influenza viruses from the North American H7 lineage, including the 2002 H7N3 viruses isolated in Chile (Suarez et al., 2004), the 2007 H7N3 viruses isolated in Canada (Hirst et al., 2004; Pasick et al., 2005; Berhane et al., 2009), and the H7 variants selected through *in vitro* adaptation (Orlich et al., 1994; Li et al., 1990; Khatchikian et al., 1989). The exact role of HA insertions generated following nonhomologous recombination in viral pathogenicity, especially in viral adaptation to mammalian hosts, has not been fully evaluated. Here, by using the Mx/7218 virus, we evaluated the role of specific basic residues in the HA insertion towards viral pathogenesis. Our study provides valuable insight into the molecular basis of the H7N3 cleavage site and its impact on the efficiency of HA cleavage, the pH of fusion, and virulence in mammals.

Results

Intracellular cleavage of Mx/7128 HA

Compared to other HA cleavage site insertions acquired by nonhomologous recombination, the insertion at the HA cleavage site of Mx/7218 virus is relatively short at 8 aa long. The aa insertion of..PENPK-**DRKSRHRR**-TR/GLF (located at positions P10-P3; shown in bold and underlined), is rich in basic residues (6 out of 8 aa are basic residues), including one ionizable histidine (H) residue at position P5 (Table 1). However, unlike the continuous stretch of basic residues (R, K) regularly seen in the HAs of HPAI (H5 or H7) viruses, this unique insertion in the Mx/7128 virus HA contains basic residues interspersed with non-basic residues. To determine whether the Mx/7218 HA can be cleaved intracellularly in the absence of exogenous trypsin, we infected 293T cells with Mx/7218 or reference H7 viruses and assessed HA cleavage. Similar to the HPAI H7N7 viruses NL/219 and NL/230, Western blot analysis showed that cell surface-expressed Mx/7218 HA was able

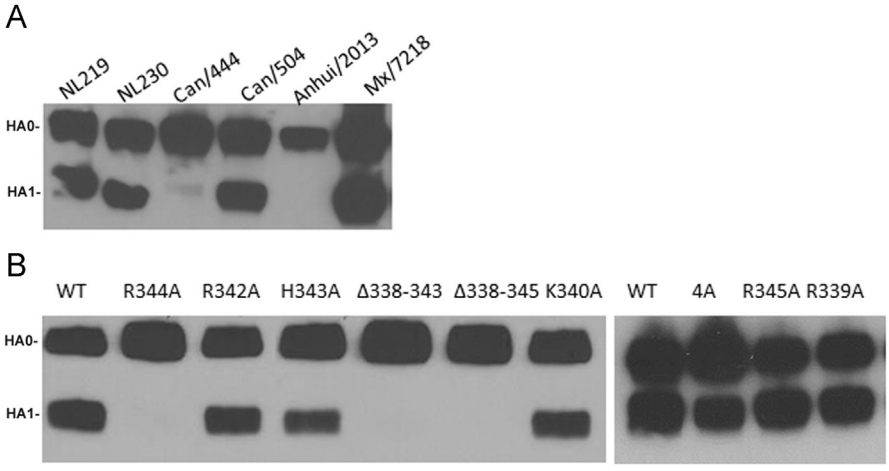

Fig. 1. The cleavability of H7 HAs in 293 T cells. The surface-expressed HAs in 293T cells from either viral infection (A) or transfection with the plasmids encoding wt or mutant forms of the Mx/7218 HA (B) were pulled down by streptavidin beads following biotin labeling and analyzed for the expression of precursor HA0 and the cleaved HA1 by Western blot with rabbit anti-HA monoclonal antibody.

Table 1
Selective H7 viruses with insertions by nonhomologous recombination at the HA cleavage site in comparison with recent HPAI or LPAI H7 viruses.

Virus	names in this study	HA sequence alignment at cleavage site [a]	origin of insertion	IVPI phenotype [b]	Genebank #	Ref
		P10 P2 P1/P1'				
A/Mexico/InDRE7218/2012	Mx/7218	PENPK**DRKSRHRRT**.....RG	host rRNA	HPAI	AFQ55689.1	Maurer-Stroh et al.,2013
A/Equine/Cornell/16/1974 (H7N7)	Eq/74	PE**NSTHKQLTHHMRKK**..... RG	unknown	unknown	ABY83099.1	Hamilton et al., 2012
A/Turkey/Oregon/1971 (H7N3) variant	Tky/71 v	PENPKT**SLSPLYPGRTTDLQVPTA**.....RG	28S rRNA (in vitro selection)	HPAI	NA	Khatchikian et al., 1989
A/Seal/Mass/1/1980 (H7N7) variant	Seal/80 v	PENPK**KEHPSAGKDPKKTGGPIYRRT** RG	NP (in vitro selection)	HPAI	NA	Orlich et al., 1994;Li et al.,1990
A/CK/Chile/4322/2002 (H7N3)	CK/Chi/4322	PENPKT**CSPLSRGRET**.....RG	NP	HPAI	AAQ77403.1	Suarez et al.,2004
A/CK/Chile/4957/2002 (H7N3)	CK/Chi/4957	PENPKT**CSPLSRGRKT**..... RG	NP	HPAI	AAQ77404.1	Suarez et al.,2004
A/Canada/444/2004 (H7N3)	Can/444	PENPK**QAYQKQM**T.....RG	M	LPAI		Hirst et al.,2004; Pasick et al., 2005
A/Canada/504/2004 (H7N3)	Can/504	PENPK**QAYQKRM**T.....RG	M	HPAI	ABI85000.1	Hirst et al.,2004; Pasick et al., 2005
A/CK/Saskatchewan/2007 (H7N3)	CK/Sas/07	PENPKT**TKPRPR**.....RG	unknown	HPAI		Berhane et al., 2009
A/Netherlands/219/2003 (H7N7)	NL/219	PEIP**KRRR**.....RG		HPAI	AAR02640.1	Neumann et al., 2010
A/Netherlands/230/2003 (H7N7)	NL/230	PEIP**KRRR**.....RG		HPAI		Neumann et al., 2010
A/AnHui/2013 (H7N9)	Anhui/2013	PEIP**KG**.....RG		LPAI	AHZ39686.1	Gabbard et al., 2014

a. the HA0 cleavage occurs at the conserved R residue at 329 (H3 numbering). On the N-terminal side of the cleavage site, the residues are denoted as P1, P2, P3, …etc, and on the C-terminal side of the cleavage site, the residues are denoted as P1',P2'… etc. The underlined sequences in bold indicate the insertions acquired by nonhomologous recombination.
b. indicates intravenous pathogenicity index test in chicken.

Table 2
Virulence of the Mx/7218 HA cleavage site recombinant viruses in mouse.

Mutant viruses	Sequence at the HA cleavage site	Stock virus titer ($log_{10}EID_{50}$/ml)	Wt loss (%)[a]	Day 3 lung titer ($log_{10}EID_{50}$/ml)[b]	Day 6 lung titer ($log_{10}EID_{50}$/ml)[b]
	339[c] 342 344 347 /348				
WT	P E N P K D R K S R H R R T R /G L F	8.75	25	6.67	6.44
R339A	P E N P K D **A** K S R H R R T R /G L F	8.75	N/D	N/D	N/D
K340A	P E N P K D R **A** S R H R R T R /G L F	7.75	N/D	N/D	N/D
R342A	P E N P K D R K S **A** H R R T R /G L F	7.75	N/D	N/D	N/D
H343A	P E N P K D R K S R **A** R R T R /G L F	8.25	N/D	N/D	N/D
R344A	P E N P K D R K S R H **A** R T R /G L F	<2	N/D	N/D	N/D
R345A	P E N P K D R K S R H R **A** T R /G L F	8.25	25	6.5	6.2
R339A/K340A/342A/H343A (4A)	P E N P K D **A A** S **A A** R R T R /G L F	8.5	25	6.5	5.4
Δ338-343	P E N P K R R T R /G L F	8.75	12.5	6.3	4.8
Δ338-345	P E N P K T R /G L F	8.5	7.09	6.6	4.6

[a] The percent mean maximum weight loss following intranasal infection with 1x10^6 EID_{50} of virus per mouse. N/D, not determined.
[b] The mean lung titer expressed by $log_{10}EID_{50}$/ml from the mice inoculated with 1x10^3 EID_{50} of virus.
[c] The numbers indicate amino acid positions starting from first methoinine.

to be efficiently cleaved (Fig. 1A). The Can/504 (H7N3) virus with an 7-aa HA insertion (..PENPK-QAYQKRM-TR/GLF) was also cleaved in mammalian 293T cells without the addition of exogenous trypsin. In contrast, the HA of Can/444 (H7N3) virus, which has an Arginine (R) to Glutamine (Q) substitution at position P4 compared to Can/504 virus, and the Anhui/2013 (H7N9) virus with a monobasic cleavage site, were not cleaved intracellularly. These data demonstrate that in the absence of exogenous trypsin, the Mx/7218 virus exhibited HA cleavability similar to that of HPAI viruses containing the typical stretch of basic residues at the cleavage site.

To better understand which residues are essential for the intracellular HA cleavage of Mx/7218 virus, we generated a series of mutations at the HA cleavage site to shorten the insertion to

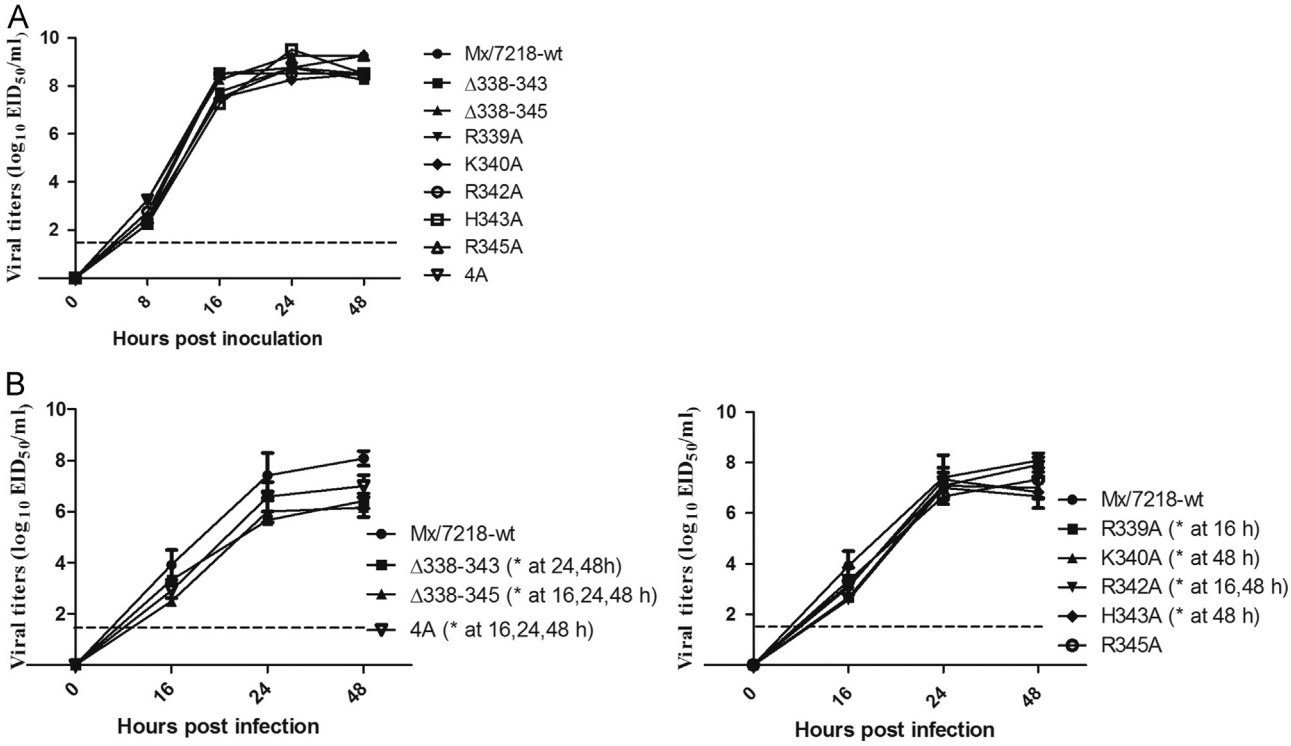

Fig. 2. Growth kinetics of the recombinant PR8 viruses with wt or mutant forms of the Mx/7218 HA *in vitro*. (A) Viral replication in chicken embryos following inoculation with 100 EID_{50} of wt or mutant Mx/7218 recombinant viruses. (B, left and right panel) Viral replication kinetics in human polarized epithelial Calu-3 cells following apical infection with the recombinant viruses at an MOI of 0.01. The viral titers from pooled allanotic fluid or infected cell supernatants were determined by titrating in eggs. Error bars represent standard deviation of viral titers from three independent infection experiments in Calu-3 cells. The asterisks in the parentheses indicate the time points at which the viral titers of the mutant viruses were significantly lower than those of the wt recombinant virus by two-way ANOVA statistical analysis ($p < 0.05$). The dotted line represents viral detection limit, which is 1.5 $\log_{10}$ EID_{50}/mL.

different lengths, or to mutate the basic residues individually or altogether (Table 2). First, we examined the cell surface-expressed HA by transfecting plasmids encoding wt or mutant HA genes. Western blot analysis showed that the total HA of wt and mutant forms were expressed at relatively similar levels, indicating that none of the mutations at the Mx/7218HA cleavage site resulted in deleterious effects on HA folding or stability (Fig. 1B). Moreover, the Mx/7218HA protein expressed from the plasmid showed a similar partially cleaved form of HA as that observed from wt Mx/7218 virus infection (Fig. 1A vs 1B). This suggested that the intracellular cleavage of Mx/7218HA in 293T cells resulted from intrinsic features of the HA gene itself, independent of NA activity, and therefore it was reasonable to assess Mx/7218HA cleavage efficiency using expression plasmids transfected into 293T cells.

First, we examined the role of the following basic residues in the intracellular HA cleavage of Mx/7218 virus; R339(-P9), K340 (-P8), R342(-P6), H343(-P5), R344(-P4) and R345(-P3) (H7 numbering starting with first methionine). We found that the alanine substitution for the histidine (H) residue at P5 position (H343A) showed a slightly reduced expression of HA1 and that alanine substitutions at P9, P8, P6, P5 and P3 have no substantial effect on the HA cleavability (Fig. 1B). In contrast, the mutant at position –P4 (R344A) resulted in no detectable cleavage of HA0 (Fig. 1B, lane 2), indicating that the R residue at P4 is required for intracellular HA0 cleavage of Mx/7218 virus. However, the R residue at position P4 was not sufficient for HA cleavage as the deletion mutant (Δ338–343) with only –RRTR/G– at the cleavage site failed to express cleaved HA1. As expected, like most low pathogenic (LPAI) H7 viruses, the deletion mutation (Δ338–345) with the –TR/G– cleavage motif expressed only uncleaved HA0 in the absence of exogenous trypsin (Fig. 1B,). Taken together, the HA insertion in the Mx/7218 virus confers the virus with intracellular cleavability in mammalian cells, and the presence of the R residue at position P4 plays a pivotal role in HA cleavage of this virus.

In vitro growth kinetics of recombinant Mx/7218 viruses with mutations at the HA cleavage site

The cleavage efficiency of HA can vary significantly depending on host-cell type and HA-activating proteases (Galloway et al., 2013; Gotoh et al., 1990). Following the observation that Mx/7218HA mutants showed varying degrees of intracellular cleavage in 293T cells, we next determined the growth of Mx/7218HA rescued viruses in two culture substrates. H7N3/PR8 reassortant viruses were generated possessing the HA and NA genes from the Mx/7218 virus and internal genes of A/Puerto Rico/8/34 (PR8, H1N1) virus. With the exception of the mutant virus harboring an alanine substitution at position P4 (–ARTR/G–), we found that the recombinant viruses with wt and mutant forms of the Mx/7218HA could be rescued and replicate to high titers in both eggs and MDCK cells. Because the mutant (–ARTR/G–) was associated with impaired viral fitness, this virus could not be included in viral replication studies. Viruses were first compared for their ability to replicate in chicken embryos where Factor Xa is thought to be the active protease (Gotoh et al., 1990). Following inoculation with 100 EID_{50}, the wt and mutant recombinant viruses exhibited similar growth kinetics in eggs; the differences in viral titers examined every 8 h p.i. were within ± 1 $\log_{10}$ and all the recombinant viruses reached comparable peak titers at 24 h p.i. (Fig. 2A). This demonstrates that neither the 8-aa insertion nor any basic residues adjacent to the HA cleavage site provides advantages in viral replication in chick embryos.

We next compared viral replication in human airway epithelial Calu-3 cells. Calu-3 cells express proteases TMPRSS2 and TMPRSS4

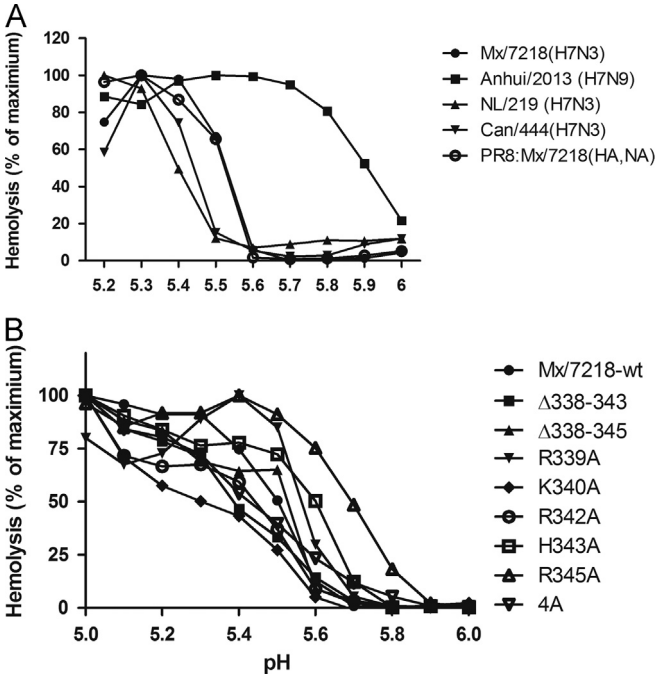

Fig. 3. The optimal pH for viral membrane fusion of Wild-type H7 viruses or recombinant Mx/7218/PR8 viruses. The virus-induced hemolysis assay was carried out by incubating viruses of 128 HAU with 2% turkey erythrocytes at 4 °C for 1 h followed by triggering fusion with 100 ul of MES buffer of varying pH values at 37 °C for 1 h. The hemolysis was measured by optical density (OD) at 405 nm and the maximal hemolysis was normalized to 100 percent. The hemolysis efficiency at various pHs was expressed as a percentage of the maximal hemolysis.

that can cleave the HA0 precursor extracellularly and support multicycle replication of influenza A viruses in the absence of exogenous trypsin (Bottcher-Friebertshauser et al., 2011). By 48 h p.i., all viruses replicated to titers greater than 6 $\log_{10}$ EID_{50}/ml, indicating that the recombinant viruses, regardless of the differences in HA cleavage site sequences, were capable of replicating to certain levels in human airway epithelial cells. However, compared to wt virus, the deletion mutant (Δ338–345) and tetra-alanine mutant (4A), exhibited reduced viral titers at all three time points (16, 24 and 48 h) examined (Fig. 2B). Conversely, the rest of mutants, except R345A showed reduced viral titers at only one or two time points (Fig. 2B). This suggests that the 8-aa insertion or the combination of four basic residues in the insertion have an important role in promoting viral replication in mammalian Calu-3 cells.

The histidine (H) at P5 and arginine (R) residue at position P3 of the Mx/7218 HA insertion modulates the optimal pH for fusion

The pH at which HA fusion occurs has been shown to affect virus stability in natural environments as well as pathogenicity and transmissibility in animal models (Imai et al., 2012; DuBois et al., 2011). A number of residues in close proximity to the fusion peptide pocket, as well as both the intra- and inter-trimer interfaces, have been shown to affect the pH for viral fusion (Steinhauer et al., 1996). Interestingly, HAs derived from human influenza viruses generally mediate membrane fusion at a lower pH range compared to the HAs of avian influenza viruses from the same subtype, suggesting that the pH for fusion might contribute to the establishment of avian viruses in new hosts (Galloway et al., 2013). To investigate whether the insertion acquired by nonhomologous recombination, affects the optimal pH for viral membrane fusion, we used a virus-induced hemolysis assay to compare the fusion pH thresholds for recombinant viruses harboring wt or mutant Mx/7218HA. Wild-type H7 viruses were included for comparison. As shown in Fig. 3A, the Mx/7218 virus could efficiently lyse turkey erythrocytes at a pH below 5.5, with ~50% of maximal fusion efficiency occurring at pH 5.5. The H7N7 (NL/219) and H7N3 (Can/444) viruses preferentially fused at a slightly lower pH of ≤ 5.4 and the H7N9 (Anhui/1) virus fused across a wider pH range (5.2–5.9) with 50% of hemolysis efficiency at pH 5.9.

Recombinant PR8 viruses harboring wt or mutant Mx/7218HA possessed similar pH fusion profiles as the wt H7 viruses (Fig. 3A vs 3B). These observations suggest that the reassortant PR8 viruses can act as a surrogate for optimal pH of membrane fusion determination. We found that the HA cleavage site insertion has no effect on viral fusion pH threshold; the recombinant viruses with either the 8-aa (Δ338–345) or 6-aa deletion (Δ338–343) showed a similar pH fusion range as the reassortant virus with wt Mx/7218HA (Fig. 3B). Interestingly, we found that both the histidine mutation (H343A) at position P5 of the cleavage site and R345 at position P3 fused at a slightly higher pH, as indicated by > 50% of hemolysis efficiency maintained at pH 5.6 (Fig. 3b). This suggests that the H residue at P5 and R residue at P3 of the Mx/7218HA cleavage site insertion may play a role in modulating the pH fusion threshold, and consequently may affect viral stability in the environment.

The 8-amino acid unique insertion is essential for Mx/7218 virulence in mice

Previous studies demonstrated that Mx/7218 virus exhibited enhanced virulence in mice and ferrets compared to phylogenetically closely related H7N3 and H7N9 viruses (Belser et al., 2013). To understand whether the insertion in the Mx/7218HA contributes to its virulence, groups of mice were inoculated with the recombinant PR8 viruses harboring wt or mutant forms of Mx/7218HA and NA (PR8:Mx/7218HA/NA). We compared the morbidity (as measured by weight loss), mortality, and virus replication following intranasal inoculation of the recombinant viruses (Fig. 4). Similar to what was observed with the 2012 wild-type Mx/7218 virus (Belser et al., 2013), the PR8:Mx/7218HA/NA recombinant wt virus (with unmodified HA) caused rapid weight loss and all inoculated mice succumbed to infection by day 6 p.i. (Fig. 4A), with no virus detected in the brain (data not shown). In stark contrast, the deletion mutant viruses HAΔ338–343 and Δ338–345, with either a dibasic residue (RRTR/G) or lacking any basic residues (TR/G) at the N terminus of the conserved R at the cleavage site, were avirulent and caused only transient weight loss in mice. These results suggest that the presence of the 8-aa insertion at the HA cleavage site is essential for the mammalian virulence of Mx/7218 virus.

Next, we investigated the role of basic residues adjacent to the cleavage site in viral virulence. As seen in Fig. 4A, the mutant virus with all four basic residues (R, K or H) from the insertion replaced with alanine (4A), and the mutant virus with an alanine substitution for R at position P3 (R345A), exhibited a similar virulence phenotype as the wt recombinant virus. All of the infected mice were euthanized by day 6 p.i. due to substantial weight loss, suggesting the basic residues located at position P3 or located further upstream of the cleavage sites are dispensable for virulence. However, due to the fact that the mutant virus with alanine substitution for R at position P4 (–ARTR/G–) failed to grow to sufficient titer *in vitro* (described above), we did not include this virus in the murine experiment and therefore cannot rule out its possible contribution in virulence.

We further compared viral replication in the lungs of mice following intranasal inoculation with 10^3 EID_{50} of the recombinant viruses (Fig. 4A). The recombinant wt virus replicated efficiently in mouse lung tissues and reached titers up to $10^{6.6}$ EID_{50}/mL on day

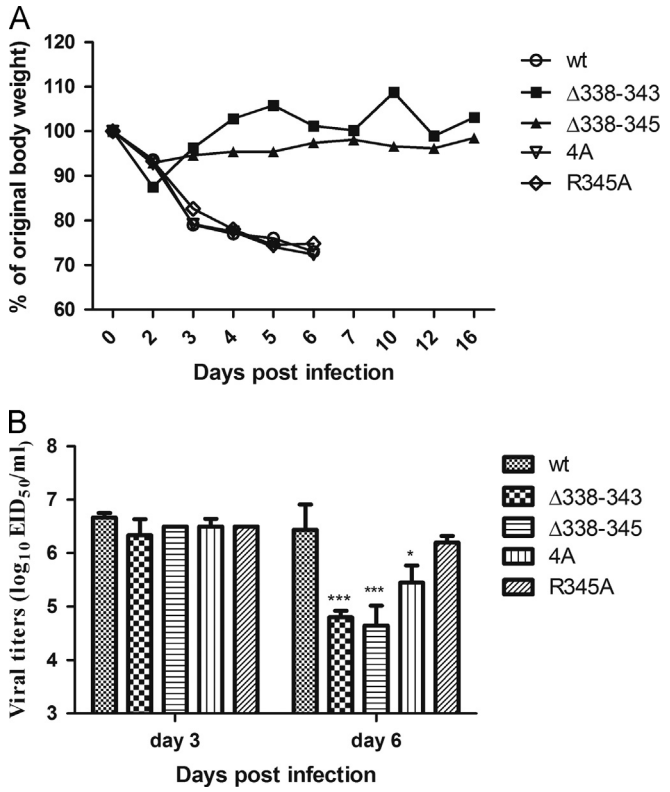

Fig. 4. Pathogenicity of recombinant Mx/7218/PR8 viruses in mice. (A) Groups of five BALB/c mice were intranasally inoculated with 10^6 EID_{50} of virus and weight loss was determined for 14 days p.i.. (B) Lungs ($n=5$) collected from mice inoculated with 10^3 EID_{50} of the indicated viruses were titered in eggs and shown as mean log_{10} EID_{50}/ml ± SD. Statistical significance between viral titers detected from mice inoculated with mutant viruses compared to the wt group was determined by two-way ANOVA and indicated by asterisks (* indicates $P<0.05$ and *** indicates $P<0.001$).

3 p.i. that persisted at elevated levels up to day 6 p.i. Compared to the wt virus, all recombinant viruses including deletion mutants (Δ338–343 and Δ338–345) or basic residue mutation viruses (4A, R345) showed no significant difference in viral replication at day 3 p.i.; however on day 6 p.i., lung titers of the non-lethal mutant viruses, HAΔ338–343 and Δ338–345, were approximately 30- and 70-fold lower respectively. Interestingly, the mutant virus (4A), which caused similar weight loss and mortality upon infection, exhibited approximately a 10-fold lower titer in the lung at day 6 p.i. (statistically different, $p<0.05$), suggesting that the basic residues in the 8-aa-insertion enhances viral replication in mouse lungs. Finally, lung viral titers of mice infected with mutant virus R345A (A substitution for R at position P3) were similar to levels of wt virus at both days p.i., indicating that the R residue at this position has no significant effect on viral replication *in vivo*. Taken together, our results suggest that the 8-aa insertion in the Mx/7218HA is essential for maintaining a lethal phenotype in mice, and the presence of basic residues upstream of position P4 contributes to efficient viral replication in the lower respiratory tract of mice.

Discussion

H5 and H7 subtype avian influenza viruses have exhibited a wide mammalian host range, leading to sporadic infection of humans. HPAI viruses typically possess a characteristic multibasic cleavage site (MBCS) in the HA, permitting the intracellular cleavage of precursor HA0 by cell-associated proteases and the systematic dissemination of viruses in multiple organs (Neumann et al., 2010). Both LPAI and HPAI H7 subtype viruses have been isolated from humans; typically LPAI H7 viruses bear a monobasic cleavage site such as the recently emerged H7N9 virus in China (Kageyama et al., 2013), and HPAI H7 viruses possess a traditional MBCS, as observed with the 2003 HPAI (H7N7) outbreak in the Netherlands (Fouchier et al., 2004). However, unlike H5 viruses, H7 influenza viruses have also demonstrated the capacity to acquire a HPAI phenotype through the acquisition of insertions by nonhomologous recombination proximal to the HA cleavage site (Hirst et al., 2004; Orlich et al., 1994; Suarez et al., 2004; Pasick et al., 2005). The sequence diversity at the HA1/HA2 boundary of H7 viruses highlights the need to better characterize the virulence determinants in the HA gene. However, most studies characterizing H7 HA cleavage sites have focused on the introduction of MBCS (Lee et al., 2006). Much less is known for the role of nonhomologous insertion in viral pathogenesis, especially in mammalian models. In this study, loss-of-function mutants were generated to investigate how the 8-aa insertion modulates Mx/7218HA cleavability and viral virulence in the mouse model. We concluded that the presence of an 8-aa insertion and the R residue at position P4 are essential for the HA intracellular cleavage and high virulence of Mx/7218 virus in mice.

At present, insertions acquired by nonhomologous recombination at the HA cleavage site have only been observed with H7 subtype viruses, primarily North American H7 lineage viruses. The mechanism of nonhomologous recombination is largely unknown, although a viral RNA polymerase slippage mechanism has been proposed by Garcia et al. (1996) and Perdue et al. (1997). Despite the great sequence variations in nonhomologous insertions, one feature shared by virtually all HA cleavage site insertions is the presence of R at position P4. Morsy et al. previously reported that the R at P4 was important for HA intracellular cleavage of A/Turkey/Oregon/71 variant Tc1 virus, which acquired an 18-aa insertion by *in vitro* selection in CEF cells (Morsy et al., 1994). In our study, we also demonstrated that the alanine substitution for the conserved R at P4 position of the Mx/7218HA completely abolished HA intracellular cleavage in 293T cells, further demonstrating that the P4-R is essential for recognition by intracellular proteases and subsequent HA cleavage. In addition, we confirmed that a Q substitution for R at P4 in Can/444/2004 virus resulted in an uncleaved HA0 (Fig. 1).

While we did not identify the exact protease(s) responsible for the Mx/7218HA cleavage in this study, we suspect that the ubiquitous furin-like proteases, which were shown to be responsible for A/Turkey/Oregon/71 variant Tc1 virus cleavage, might also be involved in Mx/7218HA intracellular cleavage (Morsy et al., 1994). Previous studies have established that furin proteases preferably cleaves the multibasic consensus motif R-X-R/K-R↓ (P4–P1 sequence) (Thomas, 2002). Structurally, the residues at P4–P1 position are packed into the furin binding pocket, and the basic residues at both P4 and P1 positions can interact with key negatively charged residues in the furin catalytic domain, providing essential binding strength between furin and the substrate (Henrich et al., 2003). However, as we demonstrate here, simply having an R at P4 and P1 positions at the HA cleavage site cannot confer HA furin cleavability, as the recombinant Mx/7218 deletion mutant virus (Δ338–343) containing only –RRTR– at the HA cleavage site lost intracellular cleavability. This suggests that additional structural requirements must be met for efficient furin cleavage. Through intensive bioinformatic analysis for possible substrates, it has been recently revealed that the furin cleavage site motif can span up to about 20 residues from P14–P6′, and furthermore the core region from P6–P2′, the surrounding regions from P7–P14, and P3′–P6′ can facilitate furin cleavage by providing a solvent accessible environment (Shiryaev et al., 2013; Tian and

Jianhua, 2010). In the case of H7 viruses bearing nonhomologous insertions at the HA cleavage site, the presence of additional polar hydrophilic amino acids upstream of the conserved R at position P4 would project the HA cleavage site further into the solvent accessible area, therefore enhancing furin cleavage. In fact, the 8aa in the Mx/7218HA insertion are either charged or polar residues that might represent a better substrate for furin compared to nonhomologous insertions seen in other H7 viruses (Table 1).

The presence of a histidine residue in the Mx/7218HA cleavage site further prompted us to investigate whether the histidine residue or even the entire 8-aa insertion affects the pH for viral fusion. Previous studies have shown that the presence of histidine residues in the insertion at the HA cleavage site of the equine/Cornell/H7N7 virus modulates both HA cleavage and viral fusion pH threshold (Hamilton et al., 2012). It has been suggested previously that certain histidine residues in the HA can function as a "histidine switch" to trigger HA conformation changes in acidic environments and prime the HA for the subsequent fusion event (Huang et al., 2003). HA proteins that fuse at lower pH tend to have improved thermostability, which might help viruses survive longer in the environment and facilitate transmission (Imai et al., 2012). In our study, we demonstrated that the Mx/7218 virus has a relatively narrow pH range for fusion compared to the newly emerged Anhui/1 (H7N9) virus, and the presence of the 8-aa insertion at the HA cleavage site has no detectable effect on the pH for viral fusion. In agreement with recently published studies (Gabbard et al., 2014), we were able to show that the Anhui/1 (H7N9) virus has a wider range of pH that will allow for fusion, approximately 5.2–5.9, although the exact role of the broad pH range for fusion in H7N9 virus pathogenesis and transmission has not been established. In our study we were able to show that the alanine substitution for the H343 (P5) showed a slightly higher pH range for fusion, suggesting the histidine at this position can stabilize the HA. Interestingly, replacing all the basic residues (4A) including H343 had no effect on viral fusion pH range. We therefore speculate that the histidine at 343 might interact with neighboring R or K located in the Mx/7218HA insertion and act synergistically in stabilizing the HA. The histidine residues in the HA insertion of the equine/76 virus have shown a similar function in stabilizing the HA (Hamilton et al., 2012).

HPAI H7 viruses exhibit a range of virulence in mammals and it has been shown that multiple viral genes contribute to their virulence in mammalian models (de Wit et al., 2010). Unlike most phylogenically related H7 viruses, the Mx/7218 virus is not only highly pathogenic in chickens, but also causes a lethal infection in mice (Belser et al., 2013). In our study, we found that the 8-aa insertion acquired by nonhomologous recombination in the HA is one of the determinants of Mx/7218 virus virulence based on the fact that the recombinant Mx/7218 virus without the HA insertion became avirulent in mice. Furthermore, we showed that the additional four basic residues (R339,K340,342,H343) upstream of position P4 in the Mx/7218HA insertion could facilitate viral replication *in vivo* as demonstrated by lower viral titers following inoculation with the 4A mutant compared to the recombinant virus containing wt HA. Although the 4A mutant HA showed no significant reduction in the HA cleavability in 293T cells compared to wt HA (Fig. 1), the recombinant viruses harboring the 4A mutation did exhibit reduced viral titers in human airway epithelial Calu-3 cells, in agreement with reduced viral titers in infected mouse lung tissues. Thus, HA cleavability is host-dependent and future studies examining the virulence of the mutant Mx/7218HA virus with the deletion or four alanine substitutions in other hosts, such as the chicken or ferret, are warranted.

Overall, this work provides critical insight into the molecular basis of the HPAI HA cleavage site as a mammalian virulence determinant. Specifically, this study demonstrates that the 8-aa cleavage site insertion acquired by nonhomologous recombination in the HA of Mx/7218 virus possess functions similar to other multibasic amino acid cleavage site motifs and contributes to the virulence of Mx/7218 virus in mice. Our work further highlights the need for increased surveillance efforts and close monitoring of avian influenza viruses with variations in the HA cleavage site. This will help identify factors that alter the virulence of avian influenza viruses and that pose health risks.

Materials and methods

Viruses, plasmids and cells

The viruses A/Mexico/InDRE7218/12 (H7N3) (Mx/7218), A/Canada/444/2004 (H7N3) (Can/444), A/Canada/504/2004 (H7N3) (Can/504), A/Netherlands/219/2003 (H7N7) (NL/219), A/Netherlands/230/2003 (H7N7) (NL/230), and A/Anhui/1/2013 (H7N9) (Anhui/1) were propagated in the allantoic cavity of 10-day-old embryonated hen's eggs at 37 °C for 26–48 h. Mx/7218 viral RNA was extracted and the HA and NA genes were cloned into the bi-directional pDZ vector as described previously (Pappas et al., 2008). The Agilent QuikChange site-directed mutagenesis kit (Santa Clara, CA) was used to introduce mutations into pDZ-Mx/7218-HA at desired sites (Table 1). A set of plasmids encoding individual internal segments of A/Puerto Rico/8/1934 (PR8) virus in pDZ vector for virus rescue was kindly provided by Adolfo García-Sastre, Mount Sinai School of Medicine, New York. Sequencing of all wild-type (wt), mutant, and rescued viruses confirmed the absence of any inadvertent mutations. Human embryonic kidney (293T) cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Madin-Darby canine kidney-London (MDCK-London) cells were obtained from the Scientific Resources Program, Centers for Disease Control and Prevention (CDC; Atlanta, GA).

HA cleavage assay

To express HA protein on the cell surface, 293T cells were either infected (multiplicity of infection (MOI)=1) for 8 h or transfected with plasmids encoding wt or mutant forms of Mx/7218HA and cultured at 37 °C for 36 h. The cell surface proteins from infected or transfected 293T cells were labeled with biotin and pulled down by streptavidin beads followed by Western blot analysis with rabbit anti-HA monoclonal antibody (11082-R002, Sino Biological Inc., China) as previously described (Sun et al., 2010).

Virus rescue, propagation and growth kinetics assay

Recombinant PR8 viruses with HA and NA genes from Mx/7218 virus and internal genes from PR8 virus were rescued from transfected 293T cells as previously described (Martinez-Sobrido and Garcia-Sastre, 2010). The recombinant virus stocks were propagated in the allantoic cavity of 10-day-old embryonated hen's eggs at 37 °C for 26 h. Viral titers were determined by inoculating eggs with serially diluted virus and the 50% egg infectious dose (EID_{50}) was calculated by the method of Reed and Muench (Reed and Muench, 1938). Viral titers were also determined by standard plaque assay in London-MDCK cells in the presence of TPCK-trypsin (1 μg/ml, Sigma, St. Louis, MO). The production of aforementioned recombinant viruses was conducted under biosafety level 3 containment, including enhancements outlined in Biosafety in Microbiological and Biomedical Laboratories (http://www.cdc.gov/biosafetypublications/bmbl5/).

To determine viral growth kinetics, groups of eggs ($n=5$) were inoculated with 100 EID_{50} of each recombinant virus and incubated at 37 °C. At every 8 h post-inoculation (p.i.), eggs were chilled and the pooled allantoic fluid was titered for the presence of infectious virus (Seladi-Schulman et al., 2013). Additionally, viral growth kinetics were assessed in polarized human airway epithelial Calu-3 cells grown on transwell inserts. Zeng et al. (2011). In brief, Calu-3 cells were inoculated with the recombinant viruses at a MOI of 0.01 for 1 h, washed, and incubated in MEM medium supplemented with 0.3% BSA at 37 °C in a 5% CO_2 atmosphere. Culture supernatants were sampled at 16, 24, and 48 h p.i. and viral titers were determined by titrating in eggs.

Influenza pH fusion threshold

Analysis of pH threshold of fusion for the recombinant H7N3 viruses was evaluated by virus induced hemolysis assay as described by Shelton et al. (2013). Briefly, viruses adjusted to 128 HAU units per 50 μl or phosphate-buffered saline (PBS) mock control were mixed with 50 μl of 2% (v/v) of turkey red blood cells and incubated at 4 °C in a 96-well plate for 1 h for maximal binding. The mixtures were pelleted at 2000 rpm for 3 min and resuspended in 100 μl of MES buffer of varying pH values (from 5.0 to 7.4) at 37 °C for 1 h to trigger hemolysis mediated by viral fusion activity. To separate nonlysed erythrocytes, the mixtures were pelleted at 2000 rpm for 5 min and the supernatants were transferred and measured by optical density (OD) at 405 nm. The OD value from mock at each pH value was subtracted to calculate hemolysis. The maximal hemolysis at a given pH was normalized to 100% and the hemolysis at other pH values was expressed as a percentage of the maximal hemolysis.

Influenza virulence in mice

All experiments using H7 viruses, including work with animals, were performed in biosecurity level-3 enhanced (BSL-3E and ABSL-3E) facilities. Animal experiments were performed under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility. Groups of fifteen 6- to 8-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were inoculated intranasally (i.n) with 10^6 EID_{50} or 10^3 EID_{50} (50 μl) of recombinant viruses diluted in PBS following anesthesia with 0.2 ml of 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co., Milwaukee, WI). Five mice per group inoculated with 1×10^3 EID_{50} of virus were euthanized day 3 or day 6 p.i. to collect lung tissues for viral titration in eggs. The remaining five mice inoculated with 10^6 EID_{50} of viruses from each group were monitored daily for weight loss and mortality. Animals that lost $\geq$25% of their pre-infection weight were humanly euthanized, in accordance with institutional animal ethics guidelines.

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